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Influence of *Pseudomonas aeruginosa* Exoproducts on Virulence Factor Production in *Burkholderia cepacia*: Evidence of Interspecies Communication

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The effect of concentrated cell-free extracellular material from stationary-phase cultures of *Burkholderia cepacia* 10661 and *Pseudomonas aeruginosa* PAO1 on virulence factor production in *B. cepacia* was assessed. While increasing concentrations of the *B. cepacia* exoproduct caused a slight increase in siderophore, lipase, and protease production in the producing organism, a significant increase in productivity was observed for all three virulence factors with the addition of the PAO1 exoproduct. Moreover, the addition of the exoproduct from a strain of *P. aeruginosa* producing reduced amounts of autoinducer caused only a slightly greater response than that of the control. Both *B. cepacia* 10661 and *P. aeruginosa* PAO1, along with two matched clinical isolates of both organisms obtained from a cystic fibrotic patient, were shown to produce variable amounts of three different types of autoinducer. The potential for interspecies signalling in microbial pathogenicity is discussed.

Burkholderia cepacia, formerly known as *Pseudomonas cepacia*, is now recognized as an important opportunistic agent of human disease (6, 8). In particular, it has received a great deal of attention owing to its increasing association with fatal pulmonary infections in patients with cystic fibrotic (CF) lung disease (9, 13). Clinically, *B. cepacia* colonization of CF patients can result in an asymptomatic carriage, a slow and continuous decline in lung function, or third, a rapid deterioration of the lung accompanied by fever, necrotizing pneumonia, and in some cases, bacteremia (6, 9). This third syndrome is not observed with other CF pathogens. Generally, it has been noted that *B. cepacia* colonizes the lung after infection by other microorganisms (24, 25). Indeed, in a recent survey of the population with CF lung disease in Greater Manchester, England, only 3.3% of patients were colonized exclusively with *B. cepacia* (11). Instead, over 80% of the patients were cocolonized with *P. aeruginosa*. Whereas *P. aeruginosa* produces a panoply of virulence factors which play an active role in the organism's pathogenicity (7, 26), little is known about the pathophysiology of *B. cepacia* (5, 14). Isolated strains of *B. cepacia* are variable in their abilities to produce hemolysins, lipase and protease (5), exopolysaccharide (2, 17), and iron-chelating siderophores (12, 21) in vitro. Consequently, there has been no direct correlation with any of these virulence factors to the organism's pathogenic status in vivo. However, a novel possibility might be the active coaggregation of *B. cepacia* and *P. aeruginosa* in the lungs, whereby one species synergistically enhances the virulence determinants of the other. To this effect, the enhancement of *B. cepacia* attachment to different surfaces by *P. aeruginosa* exoproducts has been demonstrated (1, 18).

It is now recognized that individual cells within a multicellular system can both generate signals and respond to those produced by the surrounding cells, providing a basis for cells to change in response to prevailing environmental conditions (3). In addition to physicochemical information, an individual cell can sense the local population density and maturity to synchronize growth and developmental processes within a community (23). Recently, a new subclass of response regulators, termed autoinducers, that are adapted for intercellular communication and that use *N*-acyl homoserine lactones as the signal have been identified (4, 27). These autoinducers have been shown to control and regulate a diverse range of cell-density-dependent factors in the producing organism, including virulence factor production in *P. aeruginosa* (4, 15). Since there is remarkable similarity in the structures of autoinducers produced by different bacteria (23), it is not unreasonable to suggest that chemical signals of this nature produced by one microorganism could be used by another, unrelated species to regulate gene transcription. Hence, the aim of this study was to assess the effects of *P. aeruginosa* exoproducts on virulence factor production in *B. cepacia*.

Where indicated, bacterial growth was monitored by A_{470}. Exoproducts were obtained by lyophilization of cell-free supernatants following batch culture growth of the organisms *P. aeruginosa* PAO1 and PAO-RI(pTS400) and *B. cepacia* NCTC 10661 to stationary phase (A_{470}, 1.1) in an iron-restricted, chemically defined medium at 35°C (11). The dried supernatants were resuspended in distilled water to a 50-fold concentrate, filter sterilized, and aseptically added in various amounts immediately prior to the inoculation of 20-ml cultures of *B. cepacia* in an identical chemically defined medium. Samples from the mid-exponential phase of growth (A_{470}, 0.5) were assayed for siderophore (20), protease (28), lipase (10), and autoinducer activities (19, 22).

The results shown in Fig. 1 to 3 illustrate the influence of the *P. aeruginosa* PAO1 exoproduct on siderophore, lipase, and protease production, respectively, in *B. cepacia*. In all instances, production was markedly increased by the presence of the PAO1 exoproduct. The values presented are absolute, having been corrected for carryover levels of the virulence factors assayed. Maximum levels of background activity for siderophore, lipase, and protease production by *P. aeruginosa* PAO1 in this chemically defined medium were 0.150, 0.144, and 0.210 U ml^{-1}, respectively. Moreover, these results are not

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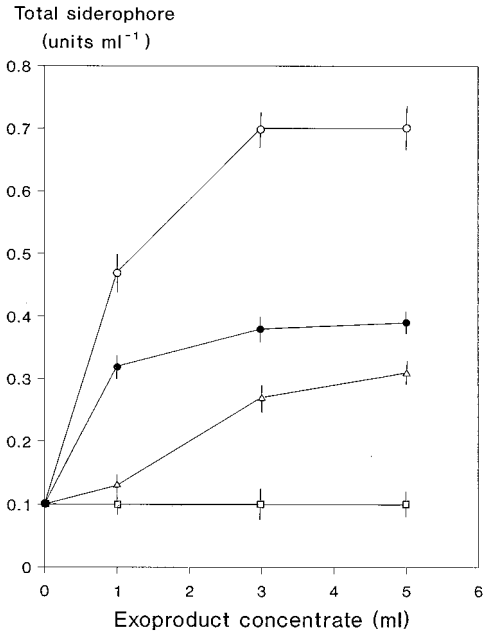

FIG. 1. Influences of the *B. cepacia*, *P. aeruginosa* PAO1, and *P. aeruginosa* PAO-RI(pTS400) exoproducts on siderophore production in *B. cepacia*. Cultures of *B. cepacia* were grown either in the absence of any exoproduct (□), in the presence of the *B. cepacia* exoproduct (△), in the presence of the *P. aeruginosa* PAO1 exoproduct (○), or in the presence of the *P. aeruginosa* PAO-RI(pTS400) exoproduct (●).

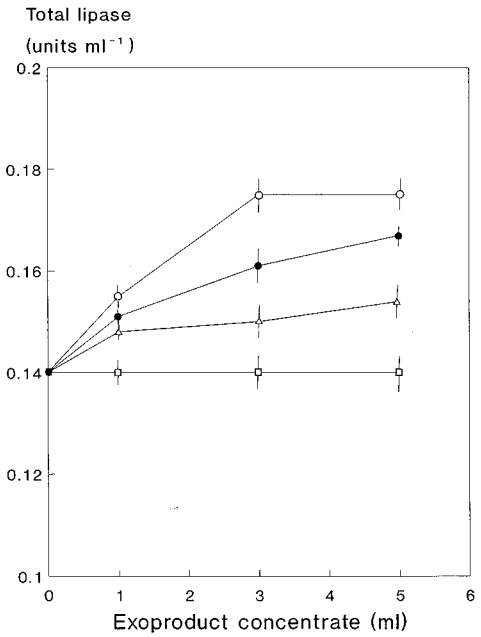

FIG. 2. Influences of the *B. cepacia*, *P. aeruginosa* PAO1, and *P. aeruginosa* PAO-RI(pTS400) exoproducts on lipase production in *B. cepacia*. Cultures of *B. cepacia* were grown either in the absence of any exoproduct (□), in the presence of the *B. cepacia* exoproduct (△), in the presence of the *P. aeruginosa* PAO1 exoproduct (○), or in the presence of the *P. aeruginosa* PAO-RI(pTS400) exoproduct (●).

due to an increase in nutrient levels, since the addition of concentrated unused growth medium to cultures of *B. cepacia* did not affect the level of virulence factor productivity (11). Siderophore production increased sevenfold with the addition of *P. aeruginosa* exoproducts up to a value of 3 ml compared with that of cultures grown in the absence of the *P. aeruginosa* material (Fig. 1). Further additions did not increase the total amount of siderophore produced. These preliminary data indicate that a compound produced by *P. aeruginosa* affects siderophore production in *B. cepacia* in a regulated manner. It is not possible to state from these results whether siderophore production occurs at a faster rate in individual cells or is switched on earlier as a result of population kinetics. The results would, however, suggest a concentration-related effect, as the amount of siderophore produced increases with increasing amounts of *P. aeruginosa* extracellular material. Since the production of siderophores enables bacteria to compete for iron with host iron-binding proteins, any mechanism which enhances siderophore production will provide a significant advantage to an infecting microorganism. This will be of particular relevance at the onset of infection when the invading pathogen is seeking to establish itself in the host. In CF lung disease, colonization by *B. cepacia* usually follows that by chronic *P. aeruginosa*, and *B. cepacia* rarely, if ever, totally replaces *P. aeruginosa* (6, 25). In this manner, *P. aeruginosa* exoproducts will be trapped in the sputum of CF patients and accumulate to a sufficiently high concentration to induce siderophore production in *B. cepacia*.

Interestingly, the addition of *B. cepacia* extracellular concentrate to growing cultures of the producing organism also resulted in an increase in siderophore production (Fig. 1). Although only a threefold increase in the level of activity was measured, this observation supports the view that siderophore production is cell density dependent. Similar results were observed for protease and lipase activities, the production of which was increased significantly by the addition of the *P. aeruginosa* exoproduct and to a lesser extent by *B. cepacia* extracellular material (Fig. 2 and 3). At high cell densities, similar to those obtained in the stationary phase of growth in a batch culture, the extracellular chemical signal accumulates to the critical concentration required for virulence factor gene transcription. Such mechanisms of signalling are consistent with those under the control of autoinducers (23, 27), with which chemicals such as homoserine lactone are produced under conditions of high cell density (15). Significantly, spent culture supernatants from strain PAO-RI(pTS400) did not produce as great a response for any of the three virulence factors assayed as those from the wild-type strain PAO1, although levels of expression were still greater than those produced by *B. cepacia* by itself (Fig. 1 to 3). Moreover, although a concentration-dependent response was again observed, the levels of expression of each virulence factor did not decrease by the same amount. Strain PAO-RI(pTS400) is a *lasR* deletion mutant derived from strain PAO1 (4) and has recently been shown to produce reduced levels of *N*-(3-oxododecanoyl)-L-homoserine lactone (OdDHL) and *N*-butyryl-L-homoserine lactone (BHL) on the orders of approximately 1,000-fold and 20-fold, respectively (16). As such, the results described in this report with PAO-RI(pTS400) culture supernatants strengthen the likelihood that the extracellular factors produced by *P. aeruginosa* are *N*-acyl homoserine lactones. Unfortunately, it has not yet proved possible to construct a stable autoinducer-minus mutant of *P. aeruginosa* (16, 26a). Furthermore, while at least four different homoserine lactones have been positively identified in *P. aeruginosa* (16, 19), their respective roles in virulence gene regulation in the parent organism, let alone in *B. cepacia*, remain to be determined. Analysis of *B. cepacia* 10661 cell-free supernatants demon-

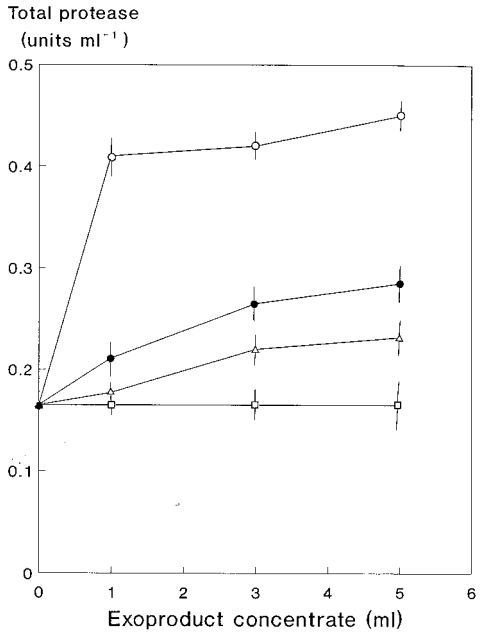

FIG. 3. Influences of the *B. cepacia*, *P. aeruginosa* PAO1, and *P. aeruginosa* PAO-RI(pTS400) exoproducts on protease production in *B. cepacia*. Cultures of *B. cepacia* were grown either in the absence of any exoproduct (□), in the presence of the *B. cepacia* exoproduct (△), in the presence of the *P. aeruginosa* PAO1 exoproduct (○), or in the presence of the *P. aeruginosa* PAO-RI(pTS400) exoproduct (●).

strated the presence of at least three types of signalling molecules, similar to those detected in *P. aeruginosa* PAO1 (Table 1). While the three reporter systems, *luxR*::*lux*, *vsmR*::*lux*, and CVO26, and associated autoinducer assays have been described and respond differentially to different autoinducers, they detect primarily *N*-(3-oxohexanoyl)-L-homoserine lactone (OHHL), BHL, and *N*-hexanoyl-L-homoserine lactone (HHL), respectively (19, 27). Hence, the strong response elicited by each sensor to the *B. cepacia* supernatant indicates the presence of OHHL, BHL, and HHL. Although the results described in this report were obtained with laboratory strains of *B. cepacia* and *P. aeruginosa*, a similar study using clinical isolates of both organisms derived from a CF patient experiencing a slow decline in lung function was performed. Almost identical trends were observed for each of the three virulence factors (11). Moreover, these clinical strains, Bc2C and Pa2C, were also shown to produce a positive, albeit lower, response in each of the three autoinducer reporter systems (Table 1). Of particular note is the observation that while all four strains activate each of the three autoinducer systems, individual strains produce different levels of response in each sensor. It is possible, therefore, that a correlation may exist between autoinducer activity (type and amount) and *B. cepacia* pathogenicity. Specific combinations of *B. cepacia* and *P. aeruginosa* may place certain CF patients more at risk of a fatal outcome than others. This is currently under investigation.

TABLE 1. Autoinducer production in *P. aeruginosa* and *B. cepacia*

Strain[a]	Relative amt of bioluminescence with autoinducer reporter system[b]:		
	luxR::*lux*	*vsmR*::*lux*	CVO26
P. aeruginosa PAO1	++	+++++	+++++
B. cepacia 10661	++++	+++++	+++
P. aeruginosa Pa2C	+	++	+
B. cepacia Bc2C	+++	++	+++

[a] Strains Bc2C and Pa2C were isolated from a cystic fibrosis patient.

[b] Plus signs represent the relative amounts of bioluminescence measured after a 4-h incubation of the test supernatants in the presence of an *Escherichia coli lux* sensor strain (with either *Photobacterium fischeri luxR* or *P. aeruginosa* PAO1 *vsmR* upstream of a promoterless *luxCDABE* cassette derived from *Photorhabdus luminescens*) or, in the case of CVO26, with the stimulation of purple pigmentation in a *Chromobacterium violaceum* inducer-negative mutant after 48 h of growth.

In conclusion, these results are extremely interesting since they provide, to our knowledge, one of the first examples of interspecies communication. Such a phenomenon will be of great importance in closely associated mixed species communities as found in CF lung tissue, in which the pathogenesis of one organism (*B. cepacia*) is affected by an unrelated organism (*P. aeruginosa*). In such communities the microorganisms exist in close proximity to each other, thereby facilitating communication and signalling between the resident cells. This chemical-signal-mediated cell-density-dependent genetic switch is likely to play an important role in other types of trapped communities, such as adherent biofilm populations.

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